Investigation of JNK/Beclin-1 Autophagy Activation in SapC-DOPS Treated Glioma

Honors Research Thesis

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Abstract

Glioblastoma (GB) is a highly aggressive brain cancer that results in poor prognosis for afflicted patients. Saposin C-dioleoylphosphatidylserine (SapC-DOPS) nanovesicle is a novel therapeutic that has been shown to cross the blood brain barrier to target glioma tumors, activate acid sphingomyelinase, induce cell death, and improve survival of mice implanted with glioma xenografts. Histological investigation of the xenograft tumors demonstrates SapC-DOPS targets the tumor vasculature and selectively induces cell death within the tumor. Co-immunoprecipitation of GB neurospheres suggests the Beclin-1/Bcl-2 interaction is involved in SapC-DOPS induced cell death. Protein analysis of SapC-DOPS treated glioma cell lines suggests autophagy is activated through lipidation of LC3-I to active LC3-II and upstream activation of JNK1 may be involved. This study evaluates the *in vivo* effects of SapC-DOPS and elucidates a possible autophagy mechanism associated with glioma cell death. Further investigation of the induced cell death pathways is required to determine the main contributors to the SapC-DOPS induced glioma cell death with the hope of forming a combination therapy that enhances SapC-DOPS efficacy and improve prognosis for glioma brain tumor patients.

Introduction

Glioblastoma (GB) is a highly aggressive and resistant stage IV primary brain tumor that encompasses 60-70% of all malignant gliomas. Current treatments combining surgical resection with adjuvant radiation and temozolomide (TMZ) are insufficient; resulting in a mean patient survival of 12-15 months.[1] Glioma patients represent a population with substantial unmet therapeutic need.

Dysregulation of tumor angiogenesis is one of the most challenging obstacles in GB therapy. Increased expression of pro-angiogenic factors such as VEGF, VEGFR, PDGF and bFGF combined with decreased expression of anti-angiogenic factors such as TSP-1, endostatin, and angiostatin increases vascular density in glioma tumors.[2] This increased blood supply provides GB tumors with sufficient serum growth factors, oxygen and waste transport to maintain larger tumor volumes.

The consequence to such uncontrolled angiogenesis, however, is leaky vasculature that causes hypoxia and inhibits the efficacy of standard GB chemo and radiation therapies. The constant proliferation of endothelial cells in response to angiogenic signaling causes degradation of the basement membrane, endothelial extracellular matrix and pericyte binding, resulting in wide, immature blood vessels that leak into the extracellular tumor microenvironment.[3] Proliferation of endothelial cells also causes bridging of endothelial cells across the lumen during blood vessel formation, leading to thrombosis. This paradoxical inhibition of blood perfusion and oxygen through pro-angiogenesis signaling creates a hypoxic tumor environment that prevents reactive oxygen species from forming during radiation therapy, while inhibited blood perfusion prevents transport of chemo therapeutics into the tumor site.[4] Areas of extreme hypoxia within

the tumor can be visualized from the presence of psuedopallisades, dense rings of glioma cells migrating away from the hypoxic microenvironment, leaving behind areas of necrosis; as is supported by *in situ* studies showing high expression of VEGF in psuedopallisade GB.[5,12]

One approach to improving glioblastoma patient prognosis is the development of novel drug therapies that selectively induce cell death in glioma cells. A vesicle-bound enzyme could potentially infiltrate the tumor and activate enzymes that signal for apoptosis, necrosis, or autophagy.[6] Saposin C (SapC) is a glycoprotein normally found within the lysosome, which facilitates in the catabolism of glycosphingolipids. SapC preferentially fuses with negatively charged phospholipids at an acidic pH, such as in the acidic tumor microenvironment caused by hypoxic conditions. When SapC is coupled with dioleoylphosphatidylserine (DOPS), stable nanovesicles are formed which preferentially fuse with cancer cells and lead to programmed cell.[7] Recent work has shown that SapC-DOPS nanovesicles specifically target phosphatidyl serine (PS) exposed on the outer leaflet of cancer cell membrane *in vitro* and *in vivo*.[8] Preliminary results on SapC-DOPS efficacy done in Kaur lab demonstrate SapC-DOPS ability to cross the blood brain tumor barrier after intravenous injection and homing of SapC-DOPS nanovesicles to the GB tumor site (Fig 1). [15]

SapC-DOPS acts by activating the ceramide producing enzyme acid sphingomyelinase once Saposin C incorporates itself into the glioma cell membrane. Acid sphingomyelinase (SMPD1) is a lysosomal and secreted enzyme that hydrolyzes sphingomyelin from the cell membrane into ceramide when activated in an acidic microenvironment.[9] Generation of ceramide has been associated with the activation of numerous apoptosis pathways that impede the progression of malignant tumor cells.[10] This association is farther supported by observed correlations between defective ceramide raft formations and radiation resistance in squamous

head and neck carcinomas.[11] Preliminary results from Kaur lab demonstrate increased survival of SapC-DOPS treated athymic nude mice with implanted with GBM xenografts (Fig 2).[15]

Interestingly, preliminary results also visualize lysosome activity in SapC DOPS treated glioma cell lines, suggesting autophagy is activated by SapC-DOPS treatment (Fig 3). Microtubule-associated protein light chain 3 (LC3) is a key signaling protein for late stage autophagy, a cell death process that utilizes lysosomes to hydrolyze intracellular components. Cytosolic LC3-I conjugates to phosphatidylethanolamine during autophagy signaling to form LC3-II, which becomes incorporated into autophagosomes.[13] This incorporation of LC3-II causes autophagosomes to engulf intracellular components and the fusion of autophagosomes with lysosomes to form autolysosomes that degrade sequestered components. This end stage of the autophagy signaling pathway causes cell death under physiologically stressful conditions.

Upstream of LC3 activity, Beclin-1 is a core protein localized in cytoplasmic structures that mediates autophagy. Beclin-1 interacts with membrane bound vascular sorting protein (vps)-15 and vps-34 to form a core protein complex that localizes autophagic proteins to the membrane and nucleates the membranes of cytoplasmic structures like the endoplasmic reticulum to form the autophagosomes required for autophagy.[14] Beclin-1 has a Bcl-2-homology (BH)-3 domain that allows inhibitory binding of Bcl-2 to Beclin-1, resulting in inhibition of beclin-1 dependent autophagy. Phosphorylation of Bcl-2 by c-Jun N-terminal kinase 1 (JNK1) has been shown to dissociate Bcl-2 from Beclin-1 to activate autophagy when JNK1 is in its active, phosphorylated state (Fig 4).

This study was designed to test the hypothesis that SapC-DOPS activates Beclin-1/Bcl-2 autophagy pathways in GB. Histological analysis determined anti-angiogenic and cell death characteristics of SapC-DOPS treated glioma xenografts. Co-immunoprecipitation of Beclin-1

elucidated freedom of Beclin1 from inhibitory protein interactions in SapC-DOPS treated GBM neurospheres. Western blot analysis of autophagy signaling protein expression determined the pathways activated in SapC-DOPS treated glioma cell lines.

Glioblastoma remains to be a challenging cancer to treat with tumor angiogenesis creating physical and chemical barriers against efficacy of the standard glioma therapy. Complications also arise from the tumor's complex dysregulation of cell death through a balance of apoptosis, necrosis and autophagy signaling. By understanding the cell death mechanisms activated by SapC-DOPS, a combination therapy with novel drug or gene therapies could enhance SapC-DOP induced cell death pathways or overcome challenges in GB tumor angiogenesis to ultimately improve glioma therapy and patient prognosis.

Methodology

Cell Culture

Human GBM cell lines obtained from ATCC. Gli36 cells were subcloned to express a truncated, constitutively active, mutant epidermal growth factor receptor (Gli36ΔEGFR), U87ΔEGFR-Luc, were obtained from Dr Webster Cavenee (Ludwig Cancer Institute, San Diego), X12 primary tumor derived cells were obtained from Dr Sarkaria, and were subcloned to express green fluorescent protein to generate X12v2 (Mayo Clinic, Rochester, MN). All cells are routinely checked for mycoplasma contamination. Cell lines were cultured with DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 10 mg/ml of streptomycin. GBM 169 neurospheres were cultured in Neurobasil media supplemented with 20 ug/ml heprin, 2 ug/ml FGF and EGF, 100 units/ml of penicillin, and 10 mg/ml of streptomycin. All cells were cultured at 37°C in an atmosphere containing 5% CO_2 and 20% O_2..

SapC-DOPS Preparation

Freeze dried SapC-DOPS for *in vitro* studies were provided by Bexion pharmaceuticals in vials of 3.6 mg SapC and 1.8 mg DOPS. Vials resuspended in 10 ml cell media produced 35 uM SapC-DOPS solution. Sub-lethal doses of SapC-DOPS were determined for each glioma cell line during protein lysate collection for western blot analysis to be 35 uM for GBM 169 and U373, 17.5 uM for X12, and 8.75 uM for gli36 glioma cell lines during 48 and 72 hours of *in vitro* SapC-DOPS treatment.

GBM Xenografts

For intracranial tumor studies, anesthetized mice were fixed in a stereotactic apparatus, and U87ΔEGFR-Luc (1 x 10^5 cells) were implanted at 2 mm lateral to bregma, at a depth of 3 mm.

Mice were treated with SapC-DOPS (SapC 1.3 mg/ml, DOPS 0.8 mg/ml, 200 ul/mouse) or PBS via tail vein injection at 5, 6, 7, 21, 22 days post tumor implantation. Tumors were excised 24h following the final treatment and harvested for immunohistochemistry analysis. Mouse brains were fixed in 4% buffered paraformaldehyde followed by 30% sucrose at 4°C, embedded in OCT, and frozen at -80°C. Tumor bearing mouse brains were sectioned in the middle of the tumor at 16 μm.

H&E Staining

Tissue slides were dried on 37°C slide warmer for 30 min, washed in PBS submersion for 10 min at room temperature, followed by a 2 min PBS wash and stained with hematoxylin for 5 sec before washing twice with DI water submersion. Slides were submerged in eosin for 1 min before washing twice with DI water, then dehydrated in 70%, 80%, 90% and 100% ethanol submersions for 5 min each and rehydrated in three 5 min xylene submersions before coverslipping in Permount.

CD31 Immunohistochemical Staining

Slides were dried on 37°C slide warmer for 30 min and washed three times in PBS submersion for 5 min at room temperature. Applied blocking solution (1:9 ratio 30% hydrogen peroxide:PBS) for 10min at room temperature, washed with PBS for 5 min, applied ABC blocking kit (Vector Laboratories SP-2001) solution A for 10 min, washed in PBS, applied solution B for 10 min, then blocked in 10% Normal Goat Serum/0.5% Triton-X in PBS for 1 hr at room temperature. Slides were treated with 1:20 rat anti-CD31 antibody (Pharmingen #550274)/0.5% Trition-X in PBS overnight in 4 °C humidified chamber. Next day washed slides three times in PBS submersion for 5 min each, applied 1:200 goat anti-rat biotin conjugated antibody/0.5% Triton-X in PBS for 1 hr at room temperature, washed three times in PBS for 10

min each, applied ABC solution for 1 hr at room temperature, and washed three times in PBS for 10 min each. Applied DAB substrate (1:9 DAB:hydrogen peroxide buffer) until development, then washed three times in PBS for 10 min and washed once in DI water for 5 min. Counter stained with hematoxylin for 5 sec, washed in DI water, dehydrated in 70%, 80%, 90% and 100% ethanol submersions for 5 min each and rehydrated in three 5 min xylene submersions before coverslipping in Permount.

TUNEL Staining

Tissue slides developed using Roche kit. Slides were dried on 37°C slide warmer for 30 min and washed twice in PBS submersion for 15 min at room temperature. Applied blocking solution (1:9 ratio 30% hydrogen peroxide:methanol) for 10min at room temperature, washed in PBS, and incubated in permeablization solution (1:9 ratio 1% Triton-X: 0.1% sodium citrate in milli-Q water) for 2 min at 4°C. Washed slides twice in PBS for 5 min, applied TUNEL kit reaction mixture and incubated 1 hr in 37°C incubator covered in humidified chamber. Washed slides three times in PBS for 5 min, applied Converter-POD solution for 30 min in 37°C, washed three times in PBS for 5 min, applied DAB substrate (1:9 DAB:hydrogen peroxide buffer) until development, then washed three times in PBS for 5 min. Counter stained with Fast Red 10 sec stain, washed in DI water, dehydrated in 70%, 80%, 90% and 100% ethanol submersions for 5 min each and rehydrated in three 5 min xylene submersions before coverslipping in Permount.

Co-Immunopercipitation

GBM 169 neurospheres were electroporated with 2 ug of FLAG-tagged Beclin-1 plasmid DNA to produce GBM Bec1 neurospheres and incubated in Neurobasil media for 24 hours. PBS washed and treated 2 x 10^6 GBM Bec1 with 35 uM SapC-DOPS or control for 48 hrs, then lysed in 200 ul precooled Sigma-Aldrich FLAG TAG lysis buffer (kit cat #FLAGIPT1). Cell solution

was incubated in 4°C shaker for 30 min, centrifuged at 12,000 g for 10 min and harvested the lysate supernatant. 40 ul ANTI-FLAG M2 affinity gel was centrifuged at 7,000 g for 1 min, incubated on ice for 2 min, supernatant was removed from resin, washed twice with 500 ul 1X wash buffer, then 500 ul elution buffer, followed by three washes with 500 ul 1X wash buffer. Cell lysates (750 ug protein), positive control (200ng FLAG-BAP fusion protein, 49.3 kDa) and negative control (lysis buffer) samples were washed in resin and filled to 1 ml lysis buffer and incubated in 4°C shaker overnight for FLAG-tagged Beclin-1 binding. Samples were then centrifuged at 7,000 g for 1 min and washed three times with 500 ul 1X wash buffer, applied 20 ul room temperature 2X sample buffer to each sample, heated each sample on 100°C block for 3 min and spun down at 7,000 g for 1 min before transferring samples to fresh eppendorf tube for western blotting.

Western Blotting

All 20 ul of co-IP samples and 15 ug of intact GBM Bec1 35 uM SapC DOPS treated and control protein lysates where denatured in 1:3 SDS solution with 4% BME and incubation on a 100°C block for 5 min, run on a 10% gel at 120 volts for 2 hrs, transferred to a cellulose membrane at 350 mA for 2 hrs, blocked in 5% BCA for 1 hr and incubated in either 1:1000 rabbit anti-FLAG (49.3 kDa control and 60 kDa Bec1), total Bec1 (60 kDa), Bcl-2 (26 kDa), or B-tubulin (55 kDa) primary antibodies in 5% BSA on 4°C shaker overnight. 15 ug of untreated or 35 uM SapC-DOPS treated U373, 17.5 uM SapC-DOPS treated X12 and 8.75 uM SapC-DOPS treated gli36 protein lysates where denatured in 1:3 SDS solution with 4% BME and incubation on a 100 °C block for 5 min, run on a 14-20% gradient gel at 100 volts for 3 hrs, transferred to a cellulose membrane at 350 mA for 2 hrs, blocked in 5% BCA for 1 hr and incubated in either 1:1000 rabbit anti-Bec1 (60 kDa), Bcl-2 (26 kDa), LC3 (14,16 kDa), JNK(46,54 kDa), and

phosphorylated JNK (46,54 kDa) primary antibodies in 5% BSA, or 1:1,000 mouse anti-GAPDH (37 kDa) primary antibody in 5% milk on 4°C shaker overnight. Membranes were washed three times with 0.1% Tween-20 in PBS for 15 min on room temperature shaker, incubated 1 hr with 1:1000 HRP-conjugated goat anti-rabbit secondary antibody in 5% BSA or 1:10,000 HRP-conjugated sheep anti-mouse secondary antibody in 5% milk on room temperature shaker, washed three times with 0.1% Tween-20 in PBS for 15 min, and developed for 5 min with ECL detection solution. HRP labeled membranes were exposed in film in a dark room cassette until protein bands were visible on the film after being processed in s film developer machine.

Results

SapC-DOPS induces cell death in Mouse Xenograft Brain Tumors *in vivo*.

To determine if SapC-DOPS *in vivo* efficacy (Fig 2) is contributed to cell death pathways, U87ΔEGFR-luc intracranial xenografts were sectioned at the center of the tumor and DNA damage was visualized using TUNEL staining. U87ΔEGFR cells are human glioma cells harboring EGFRVIII: a truncated, constitutively active, mutant epidermal growth factor receptor (ΔEGFR). EGFR amplification is the most common genetic alteration in GB and many of these tumors overexpressing EGFR also harbor the constitutively active form EGFRvIII, a strong prognostic indicator of poor survival[11]. Following treatment with SapC-DOPS, DNA damage was evident in SapC-DOPS treated U87 tumors compared to the PBS treated xenograft tumors of comparable size (Fig 5A-D). Glioma cells stained positive for TUNEL assay in SapC-DOPS treated brain sections is indicative of cell death pathways in progress, but not specific between apoptosis, necrosis or autophagy cell death pathways.

SapC-DOPS is Anti-Angiogenic against Mouse Xenograft Brain Tumors *in vivo*.

To better visualize the GB blood vessels, CD31 immunohistochemical stain was used to highlight the endothelial cells in the tumor vasculature of xenograft sections against a hematoxylin costain. PBS treated xenografts displayed numerous, dilated blood vessels that are typical for the leaky, proliferating vasculature in glioblastoma patients, while the SapC-DOPS treated xenografts had fewer and thinner vasculature structures (Fig 5E-F). Repetition and quantification of CD31 positive vessel structures in SapC-DOPS treated gli36 intracranial xenografts verified statistically significant decrease in tumor vasculature in SapC-DOPS treated

glioma xenografts (Fig 6). SapC-DOPS demonstrates significant anti-angiogenic effect in the glioma tumors *in vivo*.

Decreased Beclin1/Bcl-2 Interaction in SapC-DOPS treated Neurospheres

Bcl-2 interaction with Beclin-1 has been shown to inhibit autophagy pathways. To determine if SapC-DOPS induces autophagy by inhibition of this Bcl-2/beclin1 interaction, GBM 169 neurospheres where electroporated with DNA plasmid coding FLAG-tagged Beclin1 protein, then treated with 35 uM SapC-DOPS for 48 hours, and Beclin1 was pulled down from the protein lysates using co-immunoprecipitation beads with antibody specific for FLAG expressing protein. Interestingly, immunoblot analysis of the protein lysates showed a drastic decline in total Bcl-2 expression in both the input and immunoprecipitation protein samples while the Beclin1 protein expression remained unchanged (Fig 7). While this does not verify phosphorylation of Bcl-2 was responsible for autophagy activation, decrease in total Bcl-2 with no detectible amount of Bcl-xl or Mcl-1 suggests Beclin1 could be free to initiate autophagy after SapC-DOPS treatment.

Autophagy Activation in SapC-DOPS treated Glioma Cell Lines

Western blot protein analysis of X12, U373 and gli36 glioma cell lines treated with sub-lethal doses of SapC-DOPS demonstrate activation of late stage LC3 autophagy pathway. After 48 and 72 hours of SapC-DOPS treatment X12, U373 and gli36 cell lysates showed a dramatic drop in LC3-I expression with a modest increase in LC3-II expression (Fig 8). This observed LC3-I drop in expression is typical in autophagy activation as LC3-I is lipidated into the active LC3-II confirmation. A modest increase in LC3-II is also typical for autophagy as the active

LC3-II is degraded after fusion of the lysosomes into the autophagosome and degradation of autolysosome components. The hallmark to autophagy is the ratio of LC3-II to LC3-I, therefore quantification of the LC3-I and LC3-II band densities of the immunoblots were determined using Image J software and normalized to the GAPDH housekeeping protein band density. After SapC-DOPS treatment there is a drastic increase in glioma cell line LC3-II/I ratio that is characteristic of autophagy (Fig 9).

X12, gli36 and U373 glioma cells treated with SapC-DOPS for 48 and 72 hours exhibited no change in total JNK expression through immunoblot analysis, however an observable increase in phosphorylated JNK was observed in SapC-DOPS treated X12, gli36 and U373 cell lines (Fig 8). Increased expression of the active, phosphorylated JNK suggests involvement of Bcl-2 phosphorylation in the observed autophagy induction. No change was observed in total Beclin1 or Bcl-2 expression in the X12 cell line; however it is the interaction between Beclin-1 and Bcl-2 that is integral to autophagy regulation as well as the total Bcl-2 and Beclin-1 expression. Farther investigation of phosphorylated Bcl-2 expression and Beclin-1/Bcl-2 interaction is required to determine the pathway of autophagy induction in X12 gliomas.

Discussion

Saposin C coupled to DOPS nanovesicles have shown selective *in vivo* targeting of glioma intracranial xenografts (Fig 1) and survival efficacy (Fig 2). Previous studies from Wojton et al suggests Saposin C is capable of crossing the blood brain tumor barrier from intravenous injection and has preferential binding to phosphatidylserine overexpressed on the outer leaflet of glioblastoma cell membranes and tumor endothelial cells.[15] Once SapC-DOPS fuses with the glioma cell membrane it is hypothesized to activate endogenous acid sphingomyelinase and degrade sphingomyelin in the glioma cell membrane into ceramide. Ceramide has been proven in numerous studies to induce cell death, though the cell death pathways that are activated in glioma include cross talk of multiple mechanisms and activated cell death pathways in SapC-DOPS treated glioma are unclear.[10]

Histological analysis of U87ΔEGFR-luc and gli36 intracranial xenografts verified DNA damage that is characteristic to cell death induction after SapC-DOPS treatment and also demonstrated anti-angiogenic properties. In tumor samples of similar size, SapC-DOPS treated U87 xenografts contained more glioma cells positive for TUNEL stain and contained smaller and fewer CD31 positive blood vessel structures than PBS treated xenografts (Fig 5). Repetition of CD31 stain on a statistically significant sample size of gli36 SapC-DOPS treated xenografts verified decrease in glioma tumor blood vessel structures (Fig 6). However, TUNEL stain detecting cleavage of glioma cell DNA into exposed 3’ free endings does not distinguish between apoptotic and autophagic cell death.

Transmission electron microscopy imaging of SapC-DOPS treated glioma neurospheres visualized cytosolic vesicle formation that suggests autophagy cell death induction (Fig 3). To

determine if autophagy was activated through interaction of Beclin1 and Bcl-2, GBM 169 neurospheres with FLAG-tagged Beclin1 treated with SapC-DOPS undergone co-immunoprecipitation pull down of Beclin1 and interacting proteins were analyzed through western blotting. Interestingly, GBM 169 neurospheres exhibited decrease in total Bcl-2 expression in the input as well as the pull down protein samples (Fig 7). Decrease in Bcl-2 with no change in Beclin1 expression suggests Beclin1 is free to interact with vps-15/34 and initiate autophagy.[17] However, it cannot be concluded that Bcl-2 interaction with Beclin1 is inhibited in SapC-DOPS treated neurosphere by Bcl-2 phosphorylation due to the decrease of Bcl-2 in the SapC-DOPS input sample. Pull down of Bcl-2 and interaction with Beclin1 must be investigated to determine contribution of Bcl-2 decrease in the Beclin1 pull down is due to Bcl-2 inactivation or general destruction of cytosolic components in SapC-DOPS treated glioma.

Increase in LC3-II/I ratio in SapC-DOPS treated X12, gli36 and U373 glioma cell lines verifies induction of late stage autophagy (Fig 8). Phosphorylation of JNK is observed upstream of the Beclin1/Bcl-2 autophagy pathway by phosphorylating Bcl-2 and releasing Beclin1.[16] Increased expression of the phosphorylated form of JNK in SapC-DOPS treated X12, gli36 and U373 glioma cell lines suggests JNK cell death pathways are activated, such as the Beclin1 and Bcl-2 autophagy pathway. While no change in total Beclin1 or Bcl-2 protein expression was observed between SapC-DOPS and untreated X12 cell lines, the interaction between Beclin1 and Bcl-2 can still be altered without change in total protein expression.

While autophagy is associated with SapC-DOPS therapy, there remains the possibility that alternative cell death mechanisms in SapC-DOPS may contribute to glioma cell death. Bcl-2 is also known to interact inhibitory to pro-apoptotic factors during cell development.[16] Phosphorylation of Bcl-2 has been shown to release pro-apoptotic proteins when the cell is

stressed to produce caspases, such as caspase-9, that initiate apoptosis. The unchanged expression of Beclin1 and decreased Bcl-2 expression in the SapC-DOPS treated glioma cell lines may also support induction of apoptosis, along with autophagy activation.

While autophagy is activated in SapC-DOPS treated glioma, alternative pathways in necrosis and apoptosis must also be investigated. Increased LC3II/I ratio is hallmark to autophagy activation, but it is possible that Bcl-2 inhibited apoptosis also plays a significant role in SapC-DOPS induced cell death in glioma. It is also possible that necrosis pathways that have yet to be investigated in SapC-DOPS treated glioma could be a contributor to SapC-DOPS efficacy. Determining the mechanism behind SapC-DOPS induced cell death in glioma cell lines requires farther investigation of Beclin1/Bcl-2 interaction and possible apoptotic proteins that also have BH3 binding sites for Bcl-2. In determining the mechanism behind SapC-DOPS efficacy, a combination therapy can be devised to enhance glioma cell death and improve glioblastoma patient prognosis.

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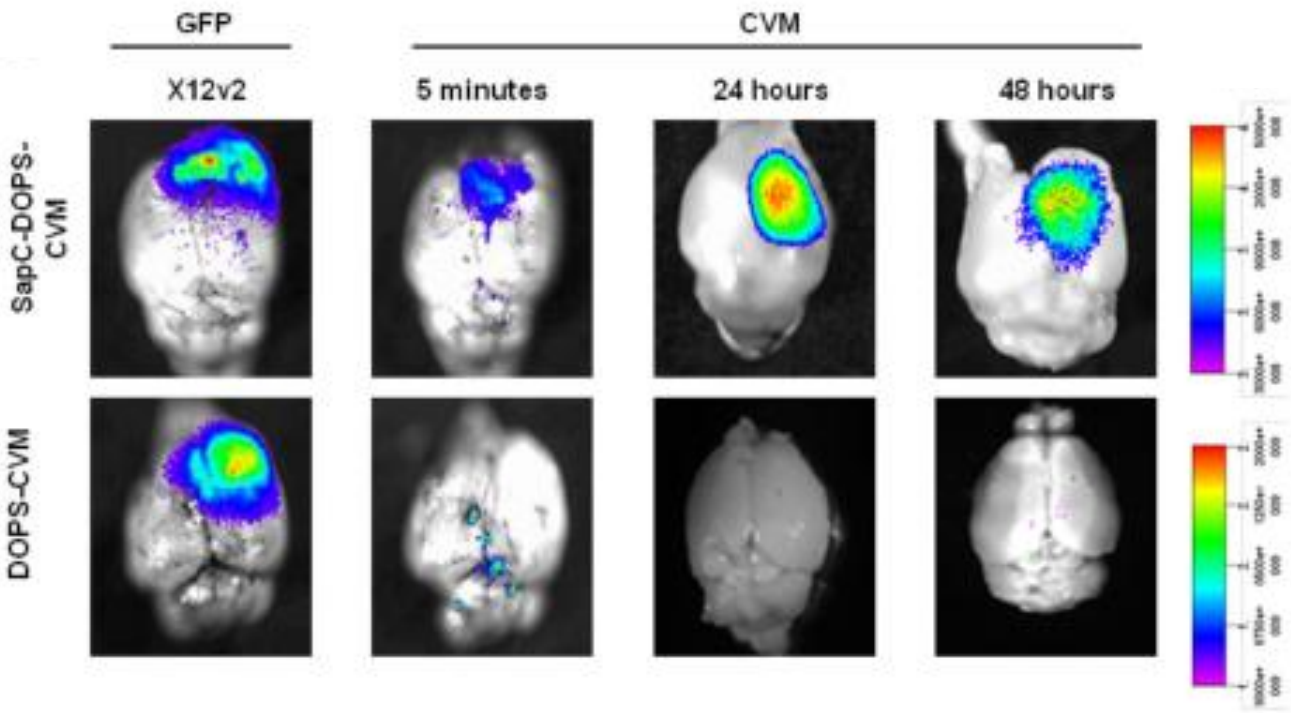

Fig (1). Fluorescence images of brains (superimposed on bright field) of mice bearing intracranial glioma (2 x 10^5 X12v2 cells) treated with intravenous SapC-DOPS-Cell Vue Maroon (SapC 12 mg/kg, DOPS 4.6 mg/kg) or DOPS-CVM (4.6 mg/kg) 10d post tumor cell implantation. SapC-DOPS passes the blood brain tumor barrier and selectively targets glioma xenografts. Image provided by graduate student Jeffery Wojton.

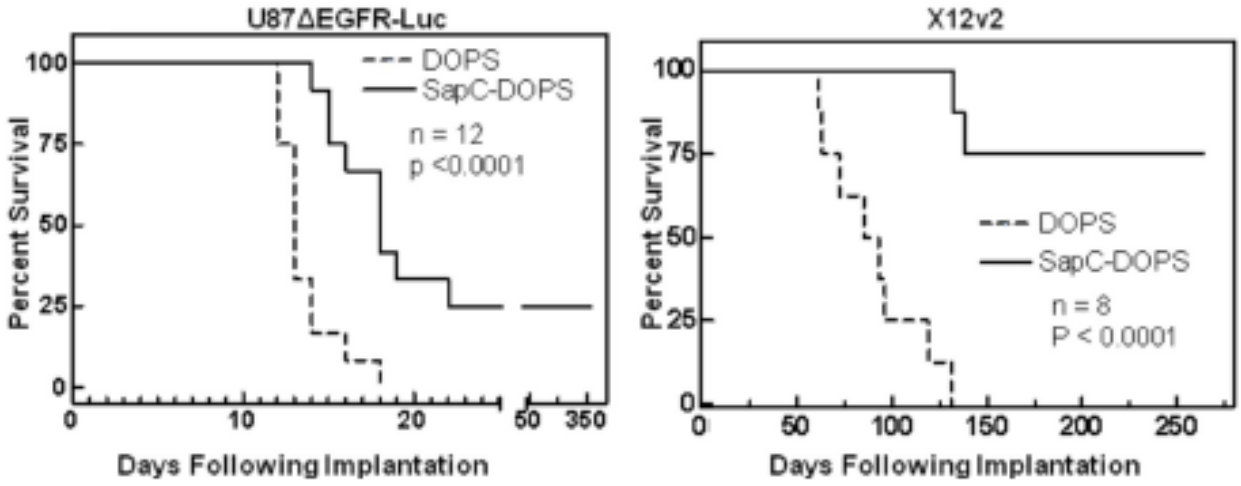

Fig (2). Kaplan-Meir survival curve for mice with intracranial U87ΔEGFR-Luc (10^5 cells) and X12v2 (2x10^5) glioma treated with intravenous injections of DOPS (4.6 mg/kg) or SapC-DOPS (SapC 12 mg/kg, DOPS 4.6 mg/kg) on days 4-11, 13, 15, 17, 19, 22, 25, 28, and 31 for U87ΔEGFR-Luc tumors and 5-9, 11, 13, and 15 for X12v2 tumors. SapC-DOPS demonstrates *in vivo* efficacy. Graph provided by graduate student Jeffery Wojton.

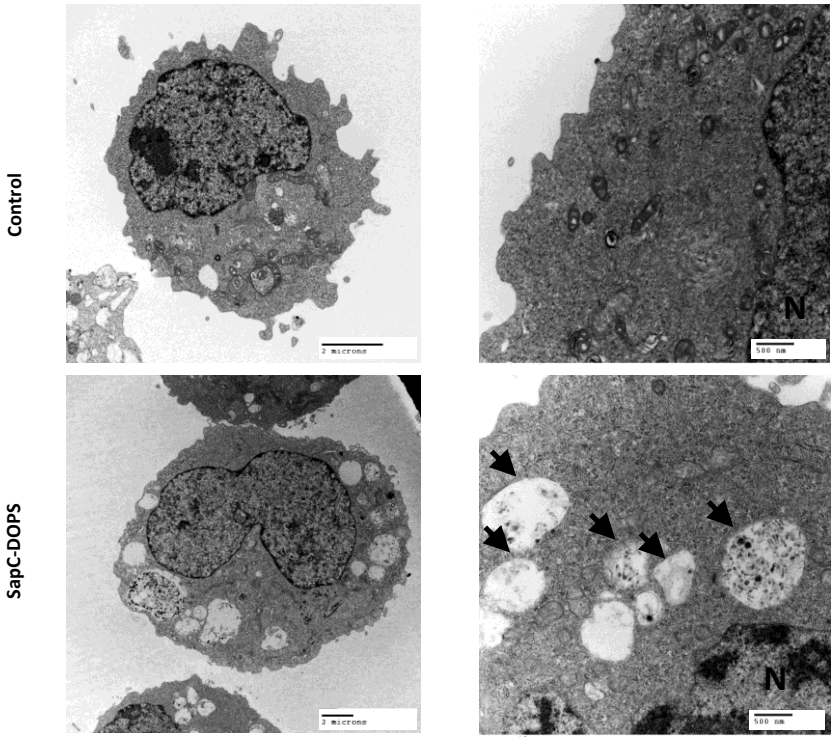

Fig (3). Transmission electron microscopy of GBM 169 neurospheres treated with SapC-DOPS (35uM) for 48h. Representative images are shown, arrow heads point to vesicles structures that are characteristic to autophagosomes. Image provided by graduate student Jeffery Wojton.

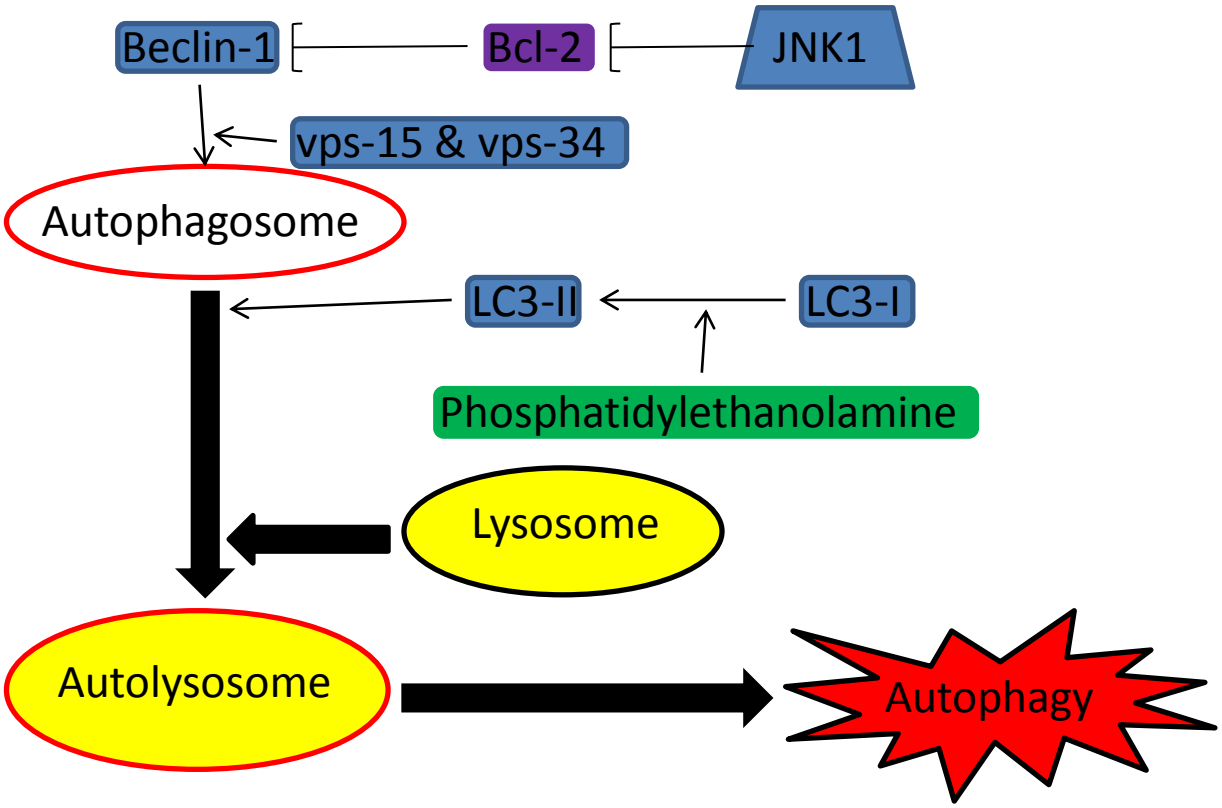

Fig (4). Schematic of proposed autophagy pathway. Beclin-1 interacts with vps-15 and vps-34 to form autophagosome from cytoplasmic structure. Beclin-1 activity is inhibited by Bcl-2 interaction, while Bcl-2 is inhibited by phosphorylation from JNK1. Incorporation of phosphatidylethanolamine into LC3-I creates LC3-II, which incorporates into autophagosomes and causes fusion of lysosomes. Resulting autolysosome hydrolyzes cytoplasm components during autophagy.

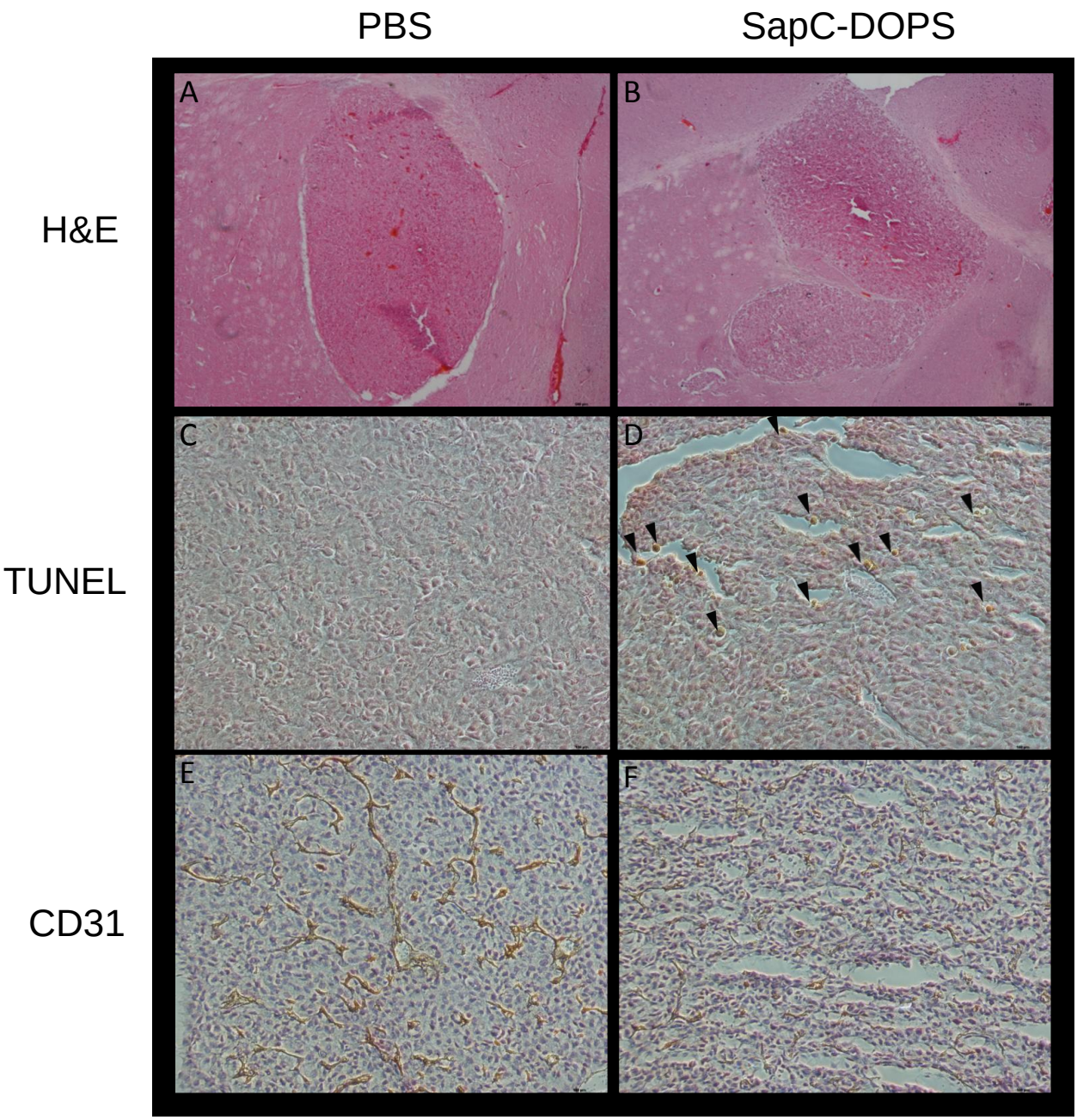

Fig (5). 4X magnification of H&E stain of U87-luc intracranial xenografts are comparable sized tumor treated with PBS (5A) or SapC-DOPS (5B). TUNEL assay of xenografts demonstrates increased cell death in SapC-DOPS treated xenografts (5D), compared to the PBS treated (5C). Decrease in CD31 positive vascular density also observed in SapC-DOPS treated (5F) compared to PBS treated (5E).

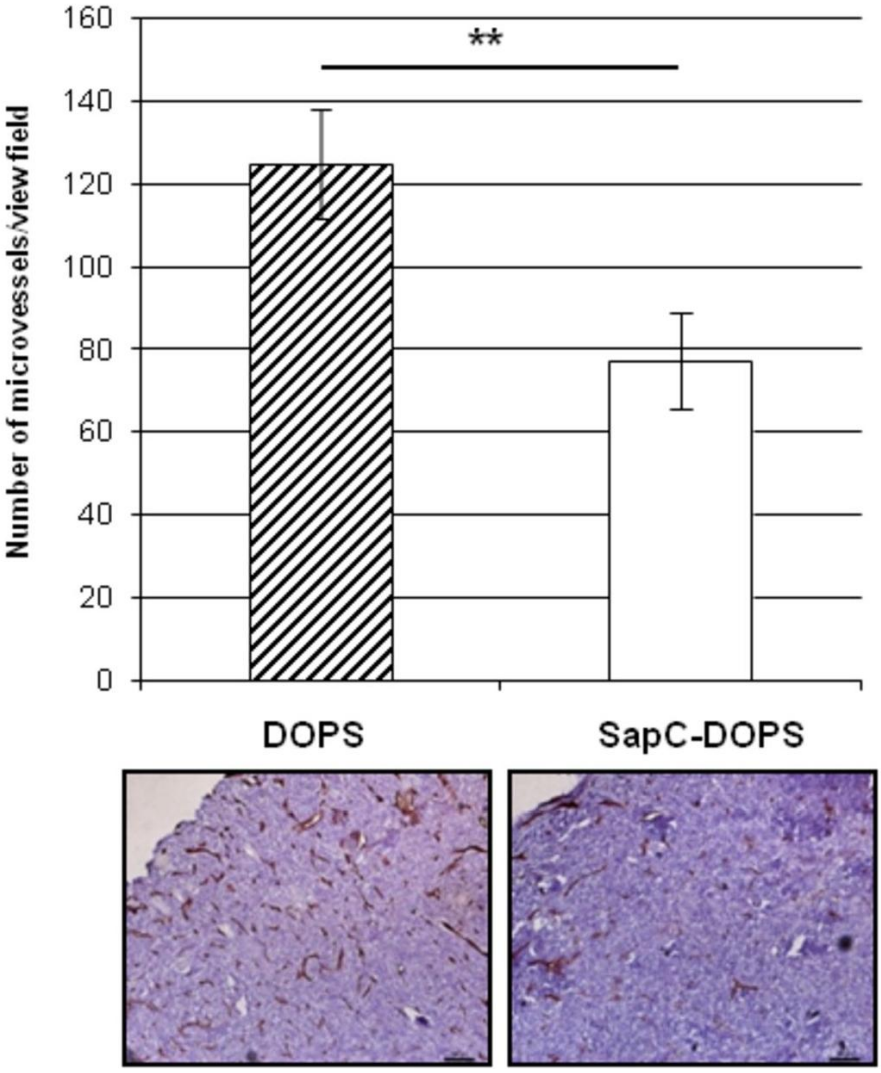

Fig (6). Repetition of CD31 staining in subcutaneous Gli36ΔEGFR tumor bearing mice were treated with 5 consecutive daily doses of SapC-DOPS (SapC 13 mg/kg, DOPS 8 mg/kg) or DOPS (8 mg/kg) by tail vein injection, and then counted vessel structures. Data shown are mean MVD ± SD for each group, n = 2 - 4 sections/tumor and n = 4 tumors/group, P = 0.001. Bottom shows representative images of tumor sections. Scale bars = 100 µM. This figure was provided by graduate student Jeffery Wojton.

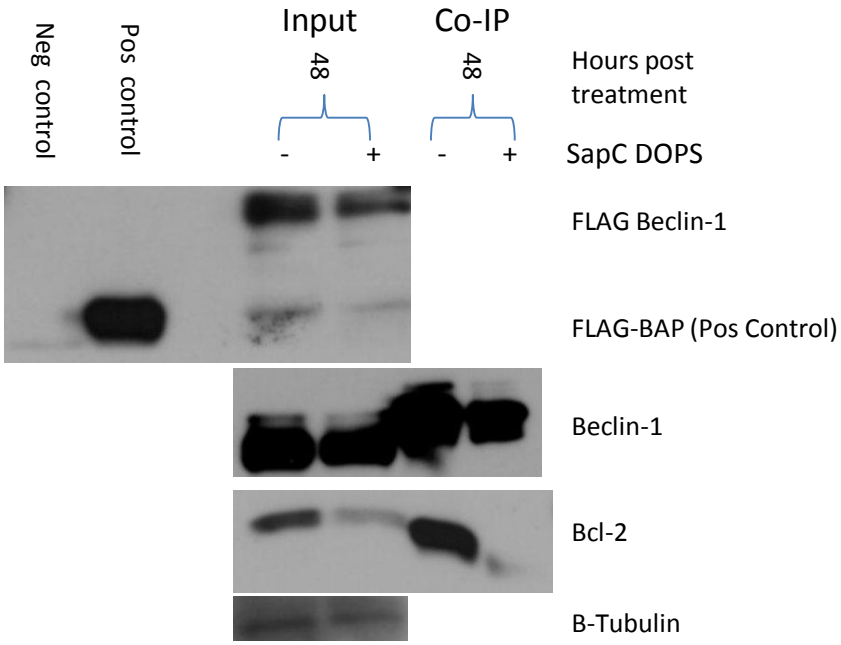

Fig (7). Immunoblot analysis of FLAG-tagged Beclin1 co-immunoprecipitation in 35 uM SapC-DOPS treated and untreated GBM 169 neurosphere protein lysates. Lysis buffer was used as negative control while FLAG-BAP acted a positive control for immunoprecipitation procedure. B-tubulin served as a housekeeping gene. After 48 hrs of 35 uM Sapc-DOPS GBM 169 neurospheres display inhibited Beclin-1/Bcl-2 interaction, but also decrease in input Beclin-1 expression.

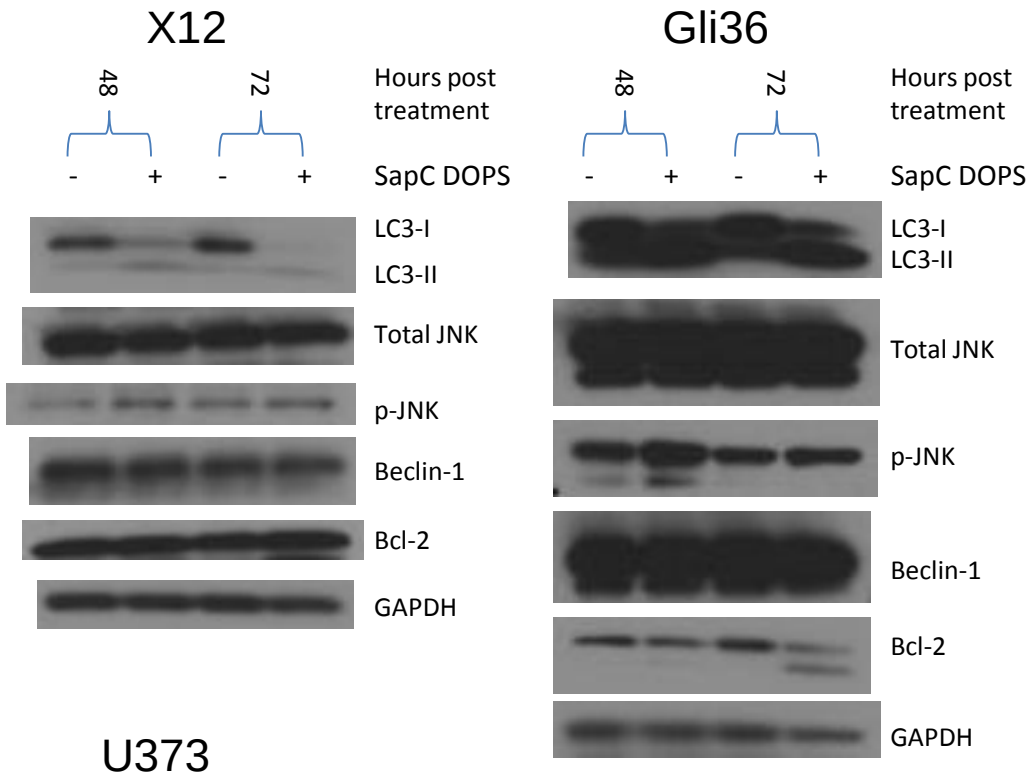

Fig (8). Immunoblot analysis of protein cell lysates of X12, gli36 and U373 glioma cell lines treated with sublethal dosage of SapC DOPS or untreated for 48 and 72 hrs. GAPDH was used as the house keeping gene. Gliomas display decreased LC3-I and increase in LC3-II and p-JNK expression after 48 hrs of SapC-DOPS treatment. Gli36 and U373 display a decrease in total Bcl-2 expression. No significant change observed in total JNK or Beclin-1 expression.

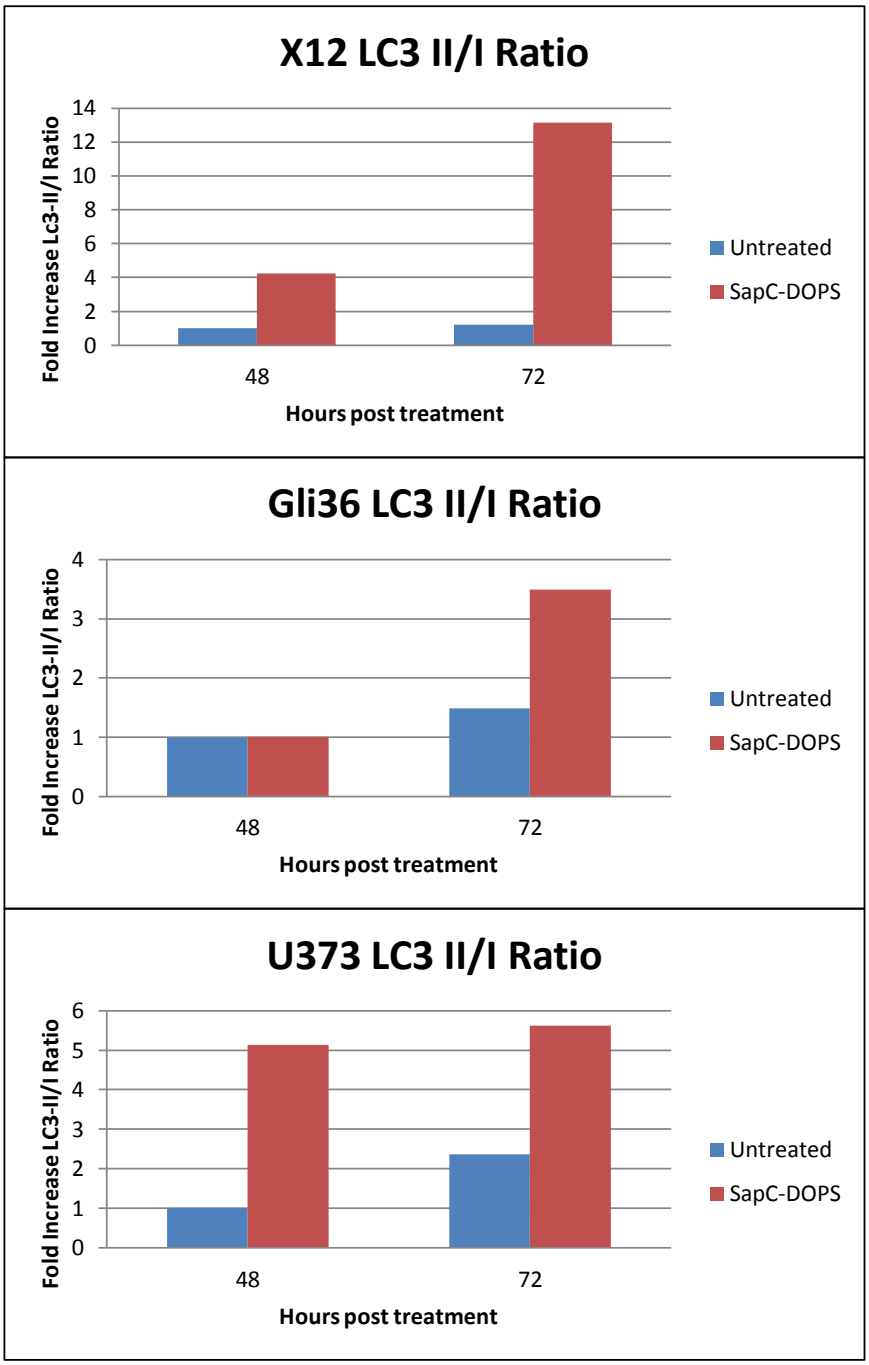

Fig (9). Quantification of X12, gli36 and U373 immunoblot LC3-II/I band density ratios. Band density quantification done using Image J software. LC3-I and LC3-II band densities for each immunoblot lane were normalized with GAPDH band density. Increase in LC3-II/I ratio under SapC-DOPS treatment is indicative for autophagy activation.